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# Biological, Physical, and Chemical Properties of Eastern Equine Encephalitis Virus

## I. Purification and Physical Properties<sup>1</sup>

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A new purification procedure was adopted for Eastern equine encephalitis virus which does not subject the virus to pelleting at any stage. Three- to 4-liter volumes were passed through a diethylaminoethyl cellulose column. The virus-containing fractions were banded on a sucrose cushion and finally concentrated in an isopycnic band in a linear sucrose gradient. This method reduced the volume 1,000-fold with a concomitant increase in viral titer, i.e., better than 90% recovery. Numerous criteria have been used to establish that this viral preparation was essentially free from cellular debris and nonviral material. Physical studies on this purified viral product were initiated. The sedimentation coefficient as determined by band sedimentation was 240S, the buoyant density in sucrose was 1.18 g/cc, and the diameter of the virus was 54 nm. From the diameter and the buoyant density it was possible to calculate the molecular weight of a spherical particle. In this case, the calculated molecular weight for Eastern equine encephalitis virus was  $58 \times 10^6$  daltons.

Two prerequisites must be fulfilled when an examination of the physical and chemical properties of a virion is attempted. The virus preparation must be free of contamination from cellular material and cell culture medium ingredients, and the concentration and purification procedures should be as gentle as possible to permit the processing of large volumes of virus-containing fluids with relative ease. We have found that concentration of various arboviruses by high-speed centrifugation does not meet these requirements. High-speed centrifugation alone, even when preceded by a low-speed sedimentation step, does not yield virus suspensions of the highest purity. In addition, sedimenting arboviruses into a tightly packed pellet appears to damage their structure. Either the physical act of pelleting or the resuspension of the pellet causes injury to the virion, possibly due to disruption of the lipoprotein membrane. Membrane damage may be observed by loss of viable titer and, in some instances, visible aggregation. The aggregation of virus particles, as well as other changes not as readily evident, presents a possible source of error in any physical study. Further-

more, a centrifugal procedure is difficult with volumes greater than 1 liter.

For these reasons, we have adopted a chromatographic procedure by employing diethylaminoethyl (DEAE) cellulose as an initial means of concentration. This procedure is a modification of that used by Fraser (*manuscript in preparation*) to purify *Escherichia coli* phages. In this report we will present a purification scheme that was developed for the preparation of high-purity Eastern equine encephalitis (EEE) virus. This scheme does not subject the virion to pelleting at any stage. Data are also presented for various physical properties of the EEE virion and permit an estimation of the particle weight.

## METHODS AND MATERIALS

**Virus and cell culture.** Sindbis virus and the Louisiana strain of EEE virus, the origin and properties of which were reported by Brown (3), were used in all experiments. Both viruses were propagated in 60-mm petri dishes by infecting washed 24-hr chick embryo cell monolayers with  $5 \times 10^6$  to  $10 \times 10^6$  plaque-forming units (PFU) of a suckling mouse brain suspension of either EEE or Sindbis virus. The inoculum was allowed to adsorb for 30 min at room temperature, the excess fluid was removed (6), and the monolayer was overlaid with 2.5 ml of Hanks Lactalbumin Hydrolysate (12) medium (HLH;

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Grand Island Biological Co.) to which 10% inactivated calf serum had been added. Virus present in the cell culture fluid was harvested after 16 hr of incubation at 37°C in a humidified incubator containing a 5% CO<sub>2</sub> atmosphere. The cell cultures were refed with 2.5 ml of HLH containing 10% calf serum, and a second harvest was made 4 to 6 hr later. The titer of EEE virus was determined by using the plaque technique described by Zehovitz and Brown (13). The titer of the first harvest ranged from  $5 \times 10^9$  to  $10 \times 10^9$  PFU/ml and that of the second about  $10^9$  to  $5 \times 10^9$  PFU/ml. The cell culture fluids containing virus were stored at -70°C.

**Virus purification.** The general purification scheme is outlined in Fig. 1. Large scale DEAE cellulose column chromatography of cell culture fluid containing EEE virus was carried out in a glass column (7 by 60 cm) enclosed in a safety cabinet. The column was packed routinely with DEAE cellulose (Eastman Organic Chemicals) suspended in a buffer containing 0.15 M NaCl and 0.15 M Na<sub>2</sub>HPO<sub>4</sub> at pH 7.5. The concentration is 60 g of DEAE cellulose to 450 ml of buffer. The column was washed with 0.05 M NaCl before use. The fluids to be processed were dialyzed overnight against 4 to 5 volumes of distilled water prior to loading on the column. After the column was charged with the virus, it was washed with 1 column volume of 0.15 M NaCl. EEE virus was eluted from the column with a buffer (TSS) containing 0.7 M NaCl, 0.08 M sodium succinate, and 0.05 M tris(hydroxymethyl)aminomethane, adjusted to pH 9.0 with concentrated hydrochloric acid. The eluate was collected in 20 ml fractions. The fractions containing the EEE virus were identified by their light scattering properties as demonstrated by shining an incandescent light beam through the virus-containing tube. The correlation between light scattering and the presence

of virus particles was confirmed by titration in cell culture of all of the column fractions.

Twenty-five milliliter samples of pooled fraction containing virus were layered over 5 ml of a 45% (w/w) sucrose solution in GNK buffer (0.05 M glycine, 0.01 N NaOH, 0.1 M KCl, pH 9.0). These were centrifuged for 2.5 hr at  $65,000 \times g$  in the no. 30 rotor of a Spinco model L ultracentrifuge. The virions were banded on top of the sucrose cushion and were removed with a pipette. The viral material was diluted 1:2 with GNK buffer. This buffer was used for all subsequent purification steps and physical studies. The virus suspension was layered on 20 ml of a 30 to 50% (w/w) linear sucrose gradient. The gradients were centrifuged for 20 hr in the SW 25 head of a Spinco ultracentrifuge at  $60,000 \times g$ . The virion band was collected by side puncture, and the virus was stored in the buffered sucrose at 4°C. The sucrose was removed from the virus suspension by stepwise dialysis against GNK buffer before any analyses were made on the virus.

**Analytical sedimentation.** A Spinco model E analytical ultracentrifuge equipped with ultraviolet adsorption optics was employed. Most experiments utilized the Vinograd band-sedimentation technique as modified by Studier (9). Buoyant density experiments were performed with a Spinco model L ultracentrifuge and a SW 25 rotor. Purified EEE virus labeled with tritiated uridine was centrifuged to equilibrium (24 hr at  $60,000 \times g$ ) in a 30 to 50% (w/w) sucrose linear gradient. Fractions, collected by puncturing the bottom of the tube, were assayed for infectivity and radioactivity. Alternate fractions were also examined with an Abbe 3-L refractometer for density determinations. Buoyant density gradients with CsCl were prepared by layering three 7-ml volumes of CsCl at densities of 1.1, 1.3, and 1.5 g/cc in a centrifuge tube. Ten milliliters of sample was then layered on top and was centrifuged at  $60,000 \times g$  for 18 hr.

**Radioactive labeling.** CE cells were labeled by adding 10  $\mu$ Ci of a <sup>14</sup>C-labeled amino acid mixture per ml and 10  $\mu$ Ci of <sup>32</sup>PO<sub>4</sub> per ml to HLH media when the cells were planted. After 20 to 24 hr, the radioactive medium was removed; the cells were washed twice with saline, removed from the petri plates, and suspended in saline. The radioactive cell suspension was then briefly sonically treated. A mixture of labeled cellular components (protein, nucleic acid, and phospholipid) and unlabeled EEE virus was prepared. This mixture was then put through the purification procedure. The presence of labeled contaminating cellular material in the virus preparation was followed during the purification procedure by assaying a 0.1-ml sample for radioactivity. Samples were prepared for counting by placing 0.1-ml samples of each fraction in a scintillation vial and adding a scintillation cocktail containing Triton X-100 (4). A Packard Tri-Carb liquid scintillation spectrometer was used for all radioactive counting. The data were corrected for spillover of <sup>32</sup>PO<sub>4</sub> counts into the <sup>14</sup>C channel.

Isotopically labeled EEE virus was prepared by infecting chick embryo cells in the normal manner

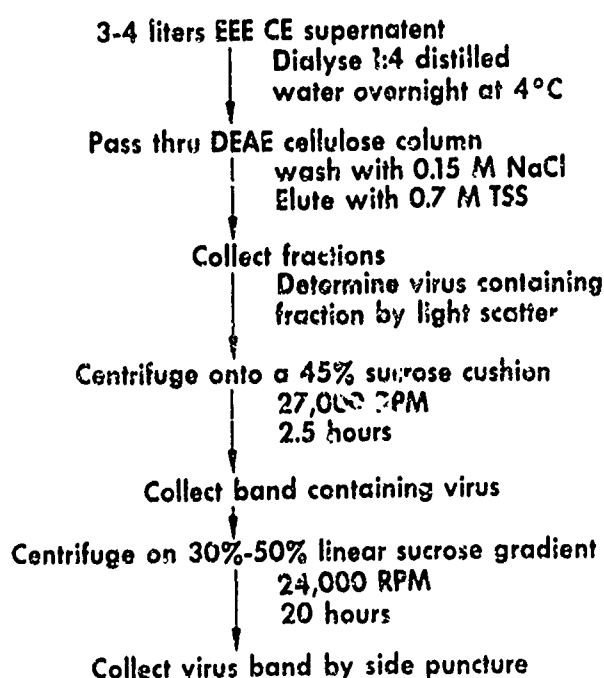


FIG. 1 Purification procedure utilized for EEE virus

and then overlaying the cells with minimal media (11) containing 5  $\mu$ Ci of tritiated uridine per ml. In those experiments in which radioactive uridine labeling of virus ribonucleic acid (RNA) was employed, actinomycin D was incorporated into the media to decrease cellular RNA formation.

**Electron microscopy.** Samples of purified virus were allowed to settle on a Formvar-coated grid. The virus was then fixed with 4% osmium tetroxide and negatively stained with 2% phosphotungstic acid (pH 5.0). In some cases, the virus was stained without being previously fixed. All electron micrographs were taken by using an RCA EMU-4 electron microscope. A diffraction grating was used to calibrate the microscope magnification factor.

## RESULTS

**Purification.** Initial experiments employing Sindbis virus were performed to adapt the DEAE cellulose column for use with arboviruses. A series of column-washing conditions was designed to determine the highest molarity of NaCl that could be used to remove weakly binding cellular debris and protein from the column without eluting the virus. The range of NaCl examined was 0.05 to 0.3 M. Considerable nonviral material was removed from the column although minimal viral infectivity was eluted with NaCl washes of up to 0.2 M. A second experiment was performed to determine the lowest molarity of eluting buffer that would quantitatively remove all of the Sindbis virus infectivity from the column.

EEE virus was chromatographed by the washing and eluting conditions described above. Three to 4 liters of dialyzed EEE virus contained in cell culture fluid was loaded onto the column. The column was washed with 1 column volume of 0.15 M NaCl and eluted with 0.7 M NaCl in TSS. The column flowed at a rate of 30 ml/min. It was observed that the virus peak consistently eluted in front of a band of red color, presumably phenol red from the HLH medium. The concentrated virus suspension represented a 20-fold increase in infectivity and a concomitant reduction in volume. Experience indicated that the DEAE cellulose performed more satisfactorily after having been used once, and for this reason all columns were cured by running through the column a batch of low titer EEE virus suspended in HLH medium. The columns were reused a number of times and were finally discarded when the flow rate was less than 15 ml/min.

We wanted to determine the amount of such cellular debris as cell protein, nucleic acid, and phospholipid which would be separated from the EEE virus by the purification technique. Therefore, we added sonically treated uninfected chick embryo cell debris in which the cellular proteins had been labeled with  $^{14}$ C-amino acids, and the

cellular nucleic acids and phospholipids were labeled with  $^{32}$ P<sub>i</sub> to tissue culture medium containing the unlabeled EEE virus to be purified. Figure 2 presents these results. Less than 10% of the original radioactivity of both radiosotopes remained associated with the EEE virus infectivity peaks. Approximately 25% of the radioactivity ran through the column as it was charged, whereas about 65% remained more tightly bound to the column than was the virus.

The next steps in purification took advantage of the size and buoyant density of the EEE virion. Those column fractions containing peak viral infectivity were pooled and 25-ml samples were layered over a 45% sucrose cushion. After high-speed centrifugation, EEE virus concentrated as a single band at the sucrose interface and contained less than 2% of the radioactivity initially placed on the sucrose cushion. EEE virus collected from the sucrose interface was very susceptible to osmotic shock and care had to be taken neither to dilute nor to dialyze the virus suspension too rapidly. Moreover, the EEE virus at this stage of purity was found to aggregate if dialyzed against tris(hydroxymethyl)aminomethane, phosphate, or borate buffers. It was determined that GNK buffer was the most effective for suspending EEE virus.

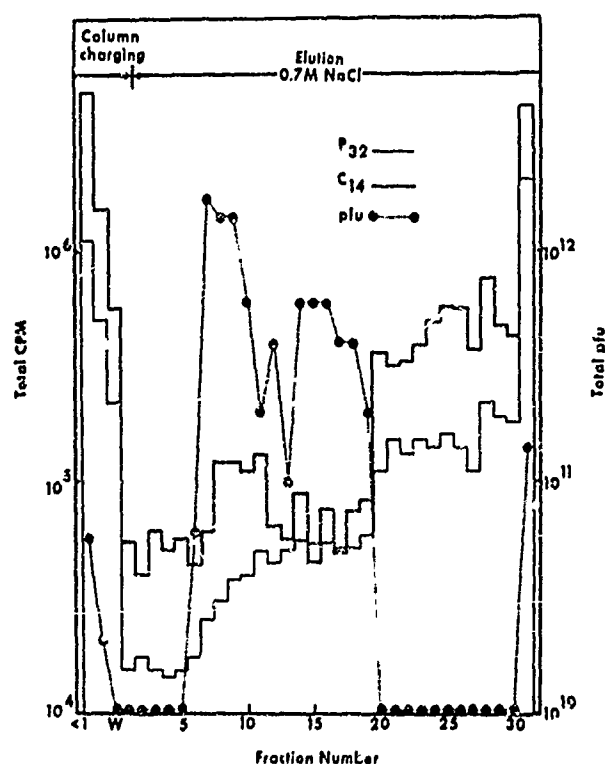


FIG. 2. DEAE cellulose column separation of labeled cellular material from unlabeled virus tissue culture preparation. The radioactivity measurements represent cellular phospholipid ( $^{32}$ P), nucleic acid ( $^{32}$ P), and protein ( $^{14}$ C-amino acids).

The final step in purification was the isopycnic banding of EEE virus in a preformed 30 to 50% linear sucrose gradient. Shown in Fig. 3 are results of an experiment in which such a gradient was fractionated by puncturing the bottom of the centrifuge tube and collecting 0.7-ml samples. This figure shows the ability of the sucrose gradient step to remove added cellular debris as evidenced by the  $^{14}\text{C}$  and  $^{32}\text{P}$  counts present in the mid and top zones of the centrifuge tube. Approximately 99% of the radioactivity remaining after the first two steps of the procedure was removed by this density gradient. Purified EEE virus maintains viability for 1 to 2 weeks when stored in GNK-buffered sucrose at 4°C.

In addition to monitoring the removal of radioactive cellular debris, another indication of the purity of the viral preparation was its optical density profile. Figure 4 shows this profile on material taken from the sucrose cushion and from the sucrose gradient. As can be seen, the sucrose cushion material has an absorption maximum at 270 nm. This indicates that there is still a significant amount of protein associated with the viral material. However, the profile of the material taken from the isopycnic sucrose gradient shows that the maximum has shifted to 262 nm. This optical density profile, similar to the profile of viral RNA, would be expected for a virus containing approximately 6% RNA and 10% protein.

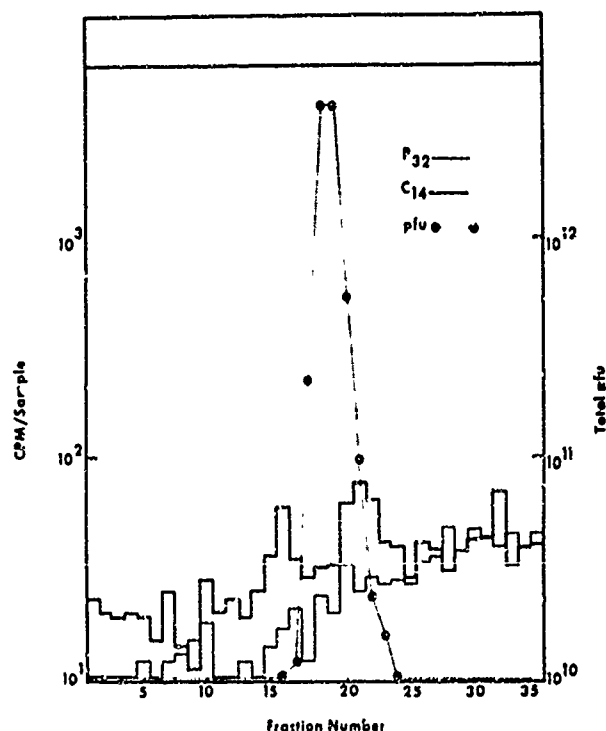


FIG. 3. Sucrose isopycnic density gradient separation of labeled cellular material from unlabeled virus particles.

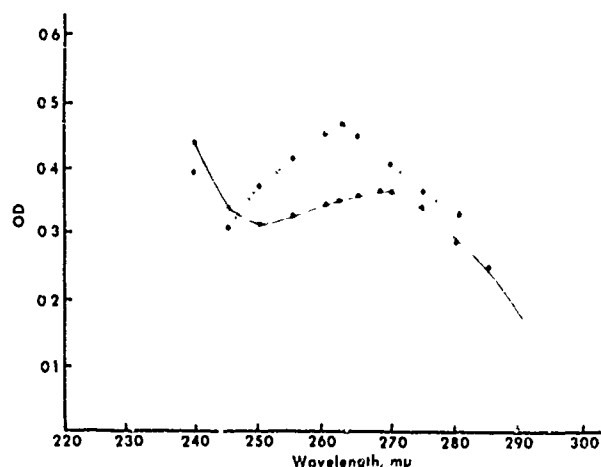


FIG. 4. Optical density profile of material taken from final two purification steps. The solid line represents material taken from sucrose cushion centrifugation with a peak at 270 nm. The broken line is the profile of material from the sucrose gradient centrifugation.

The overall efficiency of the purification scheme is summarized in Table 1. The combined concentration effect shows a volume reduction of 500-fold which in turn increases the specific infectivity to more than 16,000 times that of the starting material. The per cent recovery as PFU is greater than 100% at the final step and is probably due to an increased efficiency of plating. The actual increase in total virus is 10-fold. This increase in total virus can be duplicated by sonic treatment of the cell culture fluid in which the virus is suspended. Therefore, it appears that the purification process disrupts large clumps of virus in addition to purifying and concentrating the virus. Also included in Table 1 is the result of an isotope dilution experiment. Cellular debris labeled with  $^{14}\text{C}$ -amino acids and  $^{32}\text{P}$  was reduced by greater than 99.999% in the isopycnically banded EEE virus. Finally, there is the ratio of PFU to milligram of protein shown at each step of the procedure. There is a 10,000-fold increase over the input material, which is another indication of the removal of a considerable amount of nonviral protein.

**Physical studies.** The sedimentation coefficient of the purified EEE virion was determined by a band-sedimentation technique. An  $S_{20}^{0}$  of 240 was obtained. This figure was not corrected for viscosity and density of the medium. The tracings of the results of a typical experiment are shown in Fig. 5. Only one peak was detected in these experiments. The slight skewing of the peak to the leading edge was a characteristic of the band-sedimentation technique used.

The buoyant density of the virus was determined in a linear sucrose gradient (30 to 50%)

TABLE 1. *Efficiency of purification*

Material	Titer (PFU/ml)	Vol (ml)	Recovery of PFU (%)	Labeled cell debris (%) <sup>a</sup>	PFU per mg of protein
Raw supernatant medium <sup>b</sup>	$3.0 \times 10^9$	3,300		100	$3.0 \times 10^8$
Column eluate	$1.6 \times 10^{11}$	66	100	6	$2.7 \times 10^{10}$
Sucrose cushion	$1.5 \times 10^{13}$	3	>100	0.1	$1.1 \times 10^{12}$
Purified product	$1.9 \times 10^{12}$	6	>100	0.0007	$5.0 \times 10^{12}$

<sup>a</sup> These data are from the isotope dilution experiment; however, the titer, volume, and per cent recovery figures for the isotope dilution experiment were essentially identical to those reported in the rest of the table.

<sup>b</sup> Contains 10% calf serum.

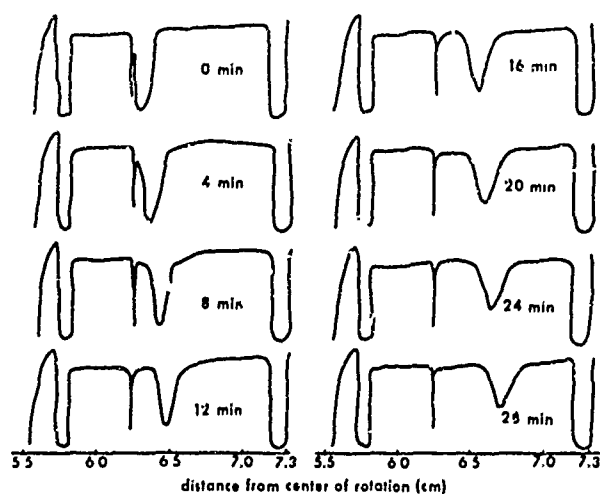


FIG. 5. Ultraviolet densitometer tracings of band centrifugation of purified EEE virus in the analytical ultracentrifuge.

which was centrifuged for 20 hr at  $60,000 \times g$  in a Spinco model L ultracentrifuge. The data in Fig. 6 show that the buoyant density of EEE virus is 1.18 g/cc. Fractions 10 to 20 of this gradient were pooled, dialyzed to remove the sucrose, and then centrifuged through a preformed CsCl gradient. The density of the infectious virus particles increased to a value of 1.20 g/cc. A radioactivity peak ( $^3\text{H}$ -uridine) was located at a density of 1.22 g/cc with a shoulder at 1.20 g/cc which confirms the results of Aaslestad et al. (1). Tubes 7 through 21 of the CsCl gradient were pooled, dialyzed, and then centrifuged on a second 30 to 50% sucrose gradient. Here again, the infectivity and radioactivity peaks were at a density of 1.17 to 1.18 g/cc. The results of this experiment are shown in Fig. 7. The second radioactive peak, found at a density of 1.07 probably represents viral degradation products resulting from the exposure to CsCl in the previous gradient.

Electron micrographs of virus particles, which had been fixed in osmium tetroxide and negatively stained, were taken (Fig. 8). Other micrographs of negatively stained unfixed virus particles were

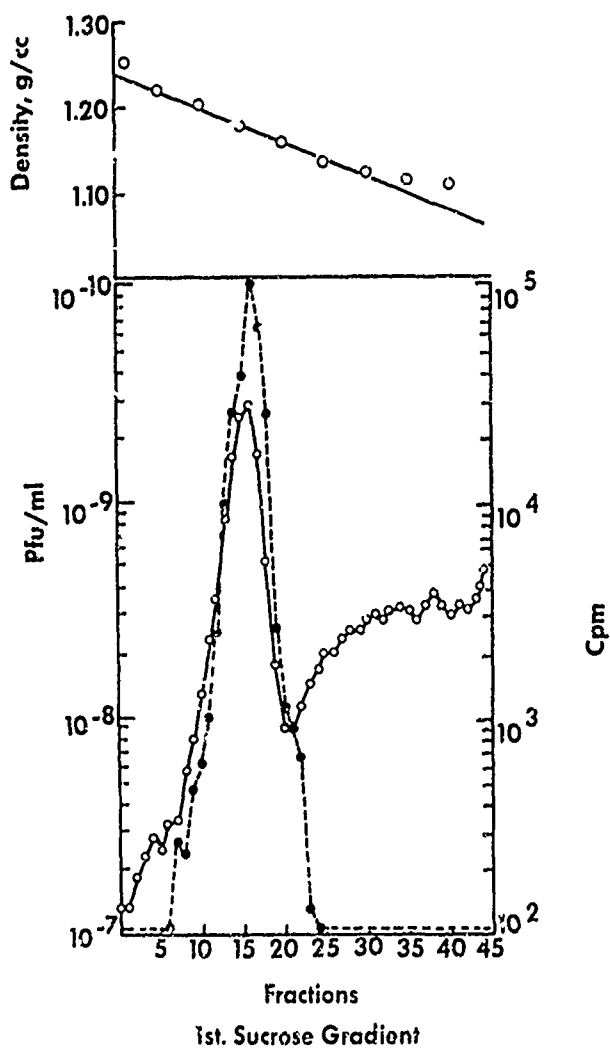


FIG. 6. Isopycnic sucrose density gradient of purified, labeled ( $^3\text{H}$ -uridine) virus particles. Closed circles represent PFU and open circles represent counts/minute. Density determinations were made on every fifth sample and the results are shown in the inset at the top of the figure.

also taken. It was determined from the measurement of a large number of particles in numerous electron micrographs that EEE is a spherical virus with diameter of  $54 \pm 5$  nm.

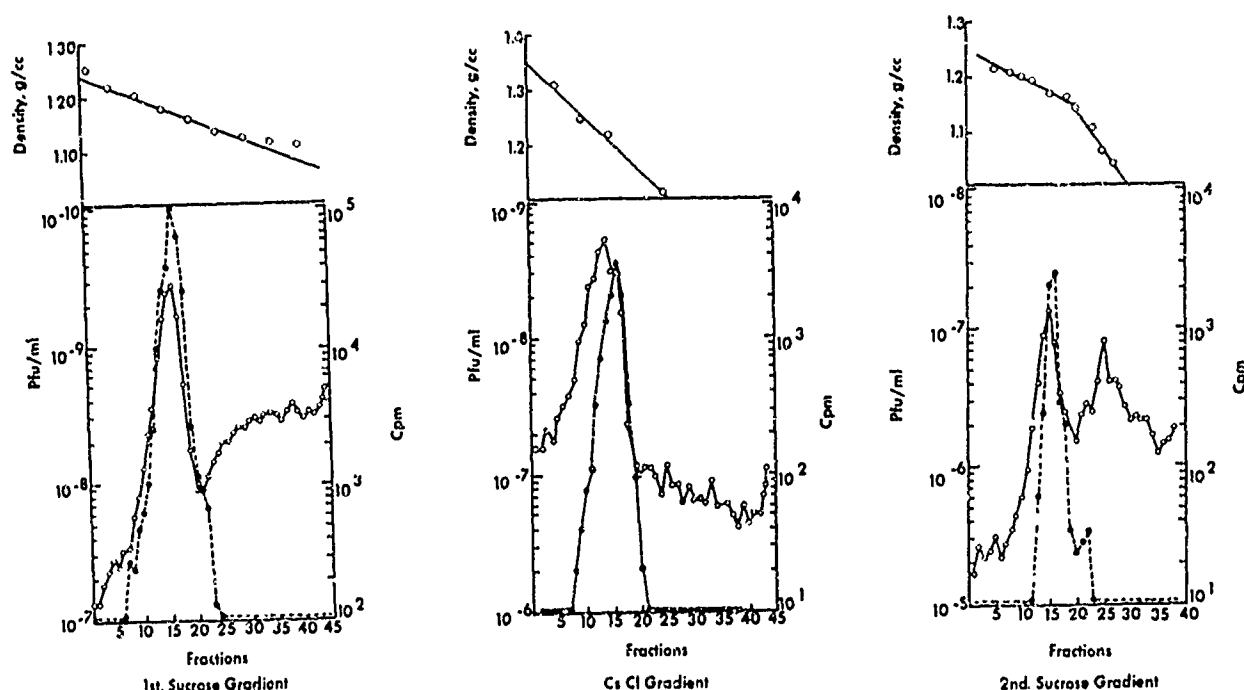


FIG. 7. Isopycnic centrifugation of EEE virus through sucrose, CsCl, and finally through a second 30 to 50% sucrose gradient. Closed circles represent PFU and open circles represent counts minute. Density determinations made as in Fig. 6.

### DISCUSSION

Purification techniques previously reported in the literature (1, 5, 8, 10) indicated that the recovery of viral infectivity varied from 15 to 60%, depending upon the technique used. With the method reported here, we routinely recovered all of the input virus. If it can be assumed that the retention of natural infectivity indicates that the essential integrity of the virus particle is not disturbed, then this technique can be assumed to be quite gentle as well as simple and highly efficient. The separation of extraneous debris from the virions is shown by the results in Fig. 2, 3, and 4 and in Table 1.

The shift in the optical density maximum (Fig. 4) would be expected with the removal of non-viral protein from the virus preparation. A virus particle which contains 6% nucleic acid, 21% lipid, and 73% protein (A. A. Fuscaldo, E. J. Hoffman, H. G. Aaslestad, and F. P. Heydrick, *manuscript in preparation*) would be expected to have maximum optical density of 258 to 262 nm since RNA has a much greater extinction coefficient than protein. The removal of nonviral protein is also indicated by the greatly increased ratio of PFU to milligram of protein (Table 1).

A description of the physical characteristics of this virus posed some difficulties. We were able to determine an  $S_{20}^0$  value for the virus. However, attempts to correct the observed  $S_{20}^0$  value for the solvent used and to have the data comparable to centrifugation through a solvent consisting of

water presented a problem. It was necessary to assume a partial specific volume ( $\bar{v}$ ) for the virus; this cannot be done with any reasonable accuracy for a particle as complex as an arbovirus. Attempting to calculate a particle weight from sedimentation data is further complicated by the fact that the  $\bar{v}$  is needed again for that calculation.

The buoyant density calculated for this virus, 1.18 g/cc, is lower than that obtained by Aaslestad, Hoffman, and Brown (1). Their experiments were carried out in CsCl, however, whereas ours were performed in a linear sucrose gradient. Aaslestad et al. also obtained three bands in their scheme, indicating that the virus particles were at least partially disrupted. Other investigators (2, 7) have also noted that lipid-containing viruses have slightly lighter densities in sucrose than in CsCl. This difference was usually attributed to differing effects of hydration in the two solutions. We have carried out experiments in which the virus buoyant density was determined in sucrose and then subsequently layered on CsCl, and the density was redetermined in this solution. The band containing most of the infectivity was then rerun on sucrose and the buoyant density was determined for a third time. A control run through three sucrose gradients was also performed. These results (Fig. 7) indicated that the difference is due to differences in hydration of the virus in the two solutions. Although the virus is disrupted by the CsCl which caused a loss in titer and the appearance of new peaks, the infective

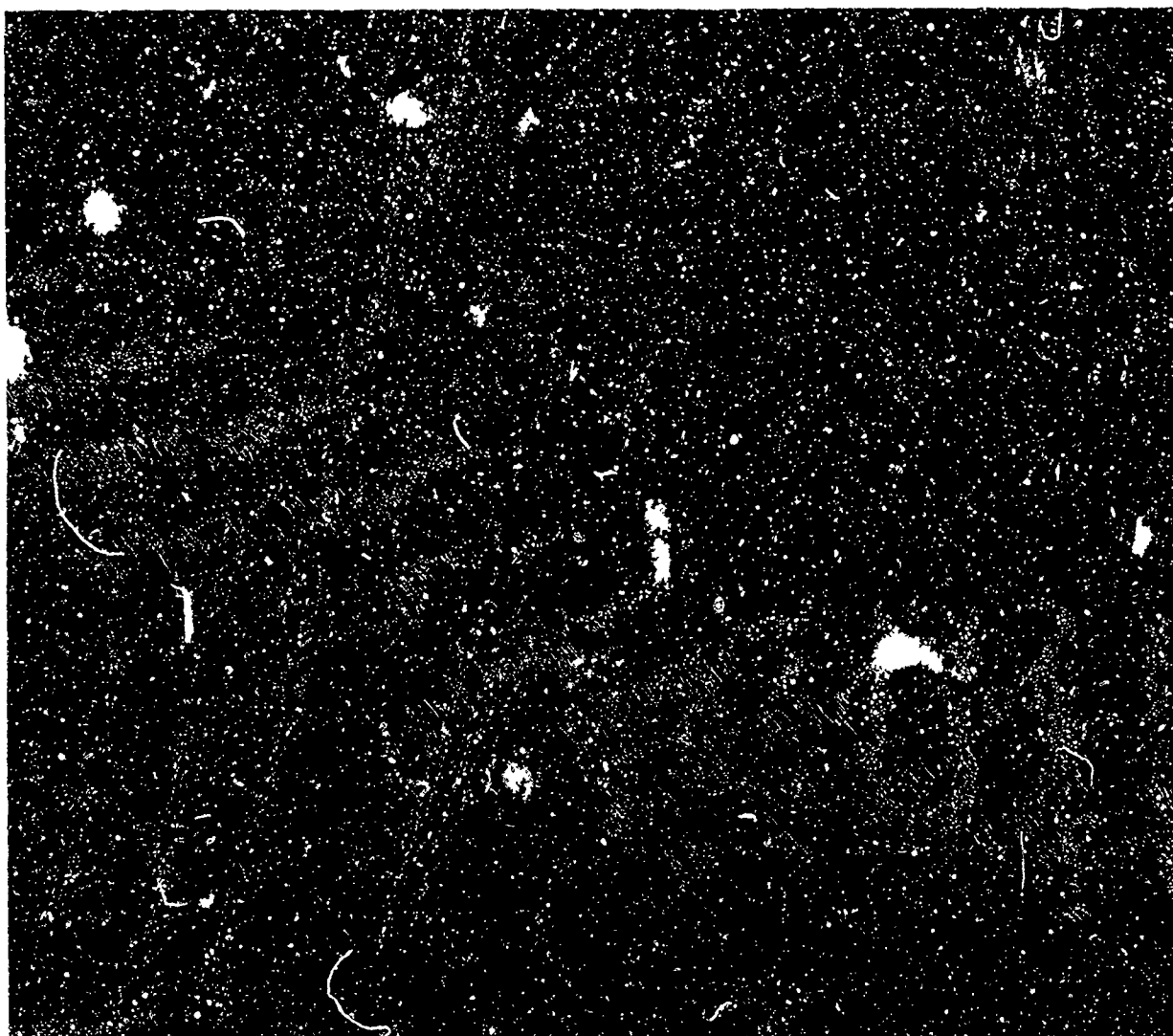


FIG. 8. Electron micrograph of EEE virus. The virus was fixed with osmium tetroxide and negatively stained with phosphotungstic acid. The line represents 0.5 nm. The magnification is  $\times 85,700$ .

virus is still located at a density of 1.17 to 1.18 g/cc in sucrose.

Since a determination of particle weight from the  $S_{20,w}$  value requires an accurate estimate of  $\bar{v}$ , we felt it impossible to make that calculation at this time. For this reason, we attempted to determine the particle weight of the virus by utilizing the volume of a spherical particle and the experimentally determined density of the virus particle. The following formula was used:  $M = (N_A D^3 \rho) / 6$  where  $N_A$  = Avogadro's number,  $D$  = diameter of the virus measured in centimeters, and  $\rho$  = the density of the virus. A basic difficulty with this formula is the assumption that the buoyant density can be substituted for the actual density. We have made this assumption on the basis that we can substitute the buoyant density into a second formula and derive a  $\bar{v}$  value which approximates the experimentally determined one. The

formula used was derived directly from Stoke's law for the coefficient of friction for a spherical body. The formula was as follows:  $S = [(\rho_v - \rho_m) / 4\pi\eta D] \times 0.426$ ; where  $\rho_v$  = density of the virus,  $\rho_m$  = density of the medium, and  $D$  = diameter of the particle in centimeter. Through the use of the above formulas, we have determined the particle weight of EEE virus to be  $58 \times 10^6$  and the calculated  $S$  value to be 227 as compared with an experimentally determined value of 240S. The calculated  $S$  value was within 10% of the uncorrected, experimental  $S$  value obtained. Furthermore, when using these formulas, close approximation to the literature values for particle weights and  $S$  values can be attained.

Now that EEE virus has been purified and concentrated to a high degree, it is possible to make a detailed analysis of the chemical composition and structure of the components. Moreover,



knowledge of the physical parameters of the intact virion should be of assistance in determining both the subparticle structure and the morphogenesis of the entire virus particle.

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